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MICELLAR LIQUID CHROMATOGRAPHY FOR THE ANALYSIS OF NUCLEOSIDES AND BASES

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SUMMARY

A new high-performance liquid chromatographic method for the analysis of nucleosides and bases was developed in which a micellar mobile phase is used. Separation was achieved on a polyvinyl alcohol (PVA) column by isocratic elution with micellar sodium dodecyl sulfate (SDS) as the mobile phase. The retention behavior of the nucleosides and bases was significantly different from that obtained by reversed-phase chromatography. Effect of pH, temperature, and concentration of SDS and the counter ion (Na^+) on retention behavior were investigated. With the PVA column, the best conditions for an isocratic separation were 0.01 *M* SDS (pH 3.4) and a flow-rate of 2 ml/min at ambient temperature. Mechanisms for the retention of the nucleosides and bases on the PVA column with a micellar mobile phase were proposed and an application of the separation was demonstrated by the analysis of human serum.

INTRODUCTION

In 1980 Armstrong and Henry[1] proposed the use of an aqueous micellar solution as a selective mobile phase in reversed-phase liquid chromatography (RPLC). This method has been used in various practical applications[2–23].

For the analysis of physiological samples containing the biologically important nucleosides and bases, high-performance liquid chromatography (HPLC) is most useful[24–32]. Most of published methods for HPLC of nucleic acid constituents involve RPLC on silica-based columns with hydroorganic mobile phases; however, it is difficult to retain some of the pyrimidines and several important compounds may not be resolved. In addition, for gradient elution, the column must be re-equilibrated, which is time-consuming.

Recently, separations of nucleosides and bases on porous polyvinyl alcohol (PVA) columns with several different buffer and salt solutions[33,34] and on a poly(styrene–divinylbenzene) (PRP-1) column with a hydroorganic mobile phase[35] have been reported. Because of relatively long retention times and poor resolution, improvements in these methods were sought. Thus, we explored the potential of micellar liquid chromatography for the separation of nucleosides and bases on a PVA column.

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EXPERIMENTAL

Apparatus

A Waters M 6000A pump (Waters Assoc., Milford, MA, U.S.A.) equipped with a Rheodyne 7125 injector (50 μl) (Berkeley, CA, U.S.A.) connected to a Waters 440 dual-wavelength detector was used. The column used was a 9-μm Asahipak GS 320H PVA column (25 cm × 7.6 mm I.D.) (Asahi Chemical Industry, Kawasaki, Japan). A Hamilton cartridge guard column (Hamilton, Reno, NV, U.S.A.) was used for protecting the analytical column. The column was placed in a constant-temperature column compartment (DuPont Instruments, Wilmington, DE, U.S.A.). Chromatograms were recorded on an Omniscribe strip chart recorder (Houston Instruments, Austin, TX, U.S.A.).

Reagents

Nucleosides and bases were obtained from Sigma (St. Louis, MO, U.S.A.). Stock solutions were prepared in double-distilled. deionized water, and the pH was adjusted to 7.3 with phosphate buffer. All stock solutions were stored at −20°C. Electrophoresis-grade sodium dodecyl sulfate (SDS) was obtained from Bio-Rad (Richmond, CA, U.S.A.) and was used as received.

Procedure

Mobile phases were prepared by adding the appropriate amount of SDS to distilled water. The pH values of the solutions were adjusted with phosphate buffer, and the solutions were filtered through 0.45-μm Nylon 66 membrane filters (Rainin Instruments, Ridgefield, NJ, U.S.A.). The separations were performed isocratically at a flow-rate of 2 ml/min at ambient temperature. Blood from a healthy volunteer was collected and centrifuged immediately. The serum was passed through a membrane cone (Amicon, Lexington, MA, U.S.A.) at 750 *g* for 15 min and stored at −20°C. Peaks were identified by previously published methods[24]. Quantitation was carried out for uridine, uric acid, xanthine, and guanosine by comparing peak heights obtained from runs on serum samples with those from runs on sets of standards. Linearity of response to each compound was obtained.

RESULTS

The retention behavior of the following nucleosides and bases was investigated: uric acid (UA), uracil (Ura), thymine (Thy), hypoxanthine (Hyp), xanthine (Xan), adenine (Ade), cytosine (Cyt), uridine (Urd), inosine (Ino), thymidine (Thd), guanosine (Guo), and adenosine (Ado). These purines and pyridines were chosen, because they are important in biological studies. The capacity factors, k', were measured as a function of the pH, the concentration of SDS, the concentration of counter ion, and the temperature.

Chromatography

Fig. 1 shows the isocratic elution profiles of nucleosides, bases, and a mixture of both classes of compounds on the PVA column when a mobile phase at pH 3.4, containing 0.01 *M* SDS, is used. Most of the hydroxylated purines and pyrimidines

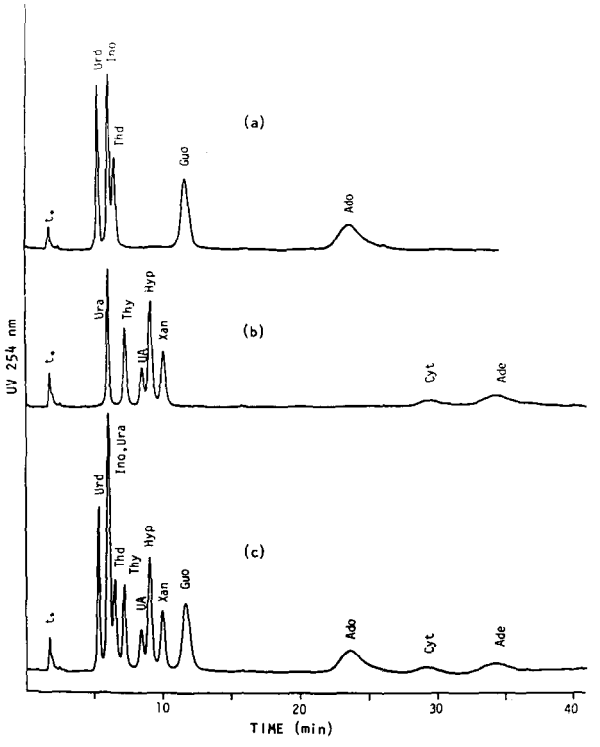

Fig. 1. Isocratic elution profiles of (a) nucleosides, (b) bases, and (c) a mixture of nucleosides and bases. Column, 9-μm Asahipak GS 320H; mobile phase, 0.01 *M* SDS (pH 3.4); flow-rate, 2 ml/min; sample concentration, $2 \cdot 10^{-5}$ *M* each; injection volume, 10 μl; sensitivity, 0.01 a.u.f.s.; temperature, ambient.

were well resolved in 12 min. Only Cyt, Ade, and Ado, compounds with amino functional groups, had longer retention times and significant band-broadening. Thus, this separation could be very useful in studies where a rapid analysis of the hydroxylated purine bases, Hyp, Xan, and UA, is needed.

Good reproducibility of retention of nucleosides and bases was observed, as can be seen in Table I. Although poor peak asymmetry is usually observed in RPLC on silica-based columns with a micellar mobile phase, we found peak asymmetry of 1.05 for Thy and 0.92 for Guo with the PVA column.

Effect of pH

The elution behaviour of nucleosides and bases on the PVA column by micellar liquid chromatography is quite different from that on RPLC silica-based columns eluted with a hydroorganic mobile phase[24–32]. With the RPLC method, the k' values of the purine bases (Hyp, Xan) were higher than those of the pyrimidine bases (Ura, Thy) in the pH range of 3.4–6, and the nucleosides had greater retention than their respective bases. However, with our method, the k' values of the purines were lower than those of the pyrimidines, and the k' values of the nucleosides (Ado, Thd, Ino, Urd) were lower than those of their respective bases (Ade, Thy, Hyp, Ura). In ad-

dition, the pyrimidine base Cyt, which is eluted near the void volume in RPLC, was strongly retained.

The elution of Ura, Thy, Urd, Ino and Thd was influenced only slightly by changes in pH from 2 to 7 (Fig. 2). On the other hand, there was a significant increase in retention at pH 3.4 for compounds possessing an amino group on the purine or pyrimidine ring (Ade, Ado, Guo, Cyt). This retention behavior is in contrast to that obtained by Yasukawa *et al.*[34] who used a phosphate buffer on a PVA column in the pH range of 3–7. They found a decrease in retention at low pH and explained that the decrease was a result of a weakening in the hydrophobic interaction of the protonated purine or pyrimidine ring moiety with the gel matrix. However, the increase in retention of Ade, Ado, Guo and Cyt at pH 3.4 that we observed can best be explained by electrostatic attraction of the protonated ring moiety with the negatively charged stationary phase. At pH 3.4, nucleosides and bases with an amino group are positively charged[37–39]. Since negatively charged sites are produced by adsorption of the anionic surfactant monomers on the surface of the PVA stationary phase, these negatively charged sites can cause the positively charged ring moiety to be retained longer than other species. Therefore, the PVA stationary phase in the presence of SDS can act as a cation exchanger. A possible retention model of the electrostatic attraction between the positively charged solute and a negatively charged stationary phase site is shown in Fig. 3.

Effect of SDS concentration

The concentration of SDS in the mobile phase at pH 3.4 affected the retention mainly of Ade, Ado, Guo and Cyt, which have an amino group on the ring. The k' values of these compounds decreased greatly as the concentration of SDS increased (Fig. 4). This increase in retention can be explained by the formation of anionic micelles of the excess SDS molecules in solution; thus, there is electrostatic attraction of the positively charged ring moiety to the increased number of anionic micelles in the mobile phase. The positively charged rings compete effectively with the counter

TABLE I

REPRODUCIBILITY OF RETENTION OF NUCLEOSIDES AND BASES

All conditions as in Fig. 1. $n = 10$.

Compound	k'	*S.D.*	*C.V. (%)*
Urd	2.12	0.00882	0.416
Ino	2.54	0.0129	0.508
Ura	2.54	0.0129	0.508
Thd	2.82	0.00882	0.313
Thy	3.24	0.0137	0.423
UA	4.00	0.0149	0.373
Hyp	4.27	0.0176	0.412
Xan	4.92	0.0180	0.366
Guo	5.77	0.0191	0.331
Ado	12.5	0.0943	0.754
Cyt	15.8	0.111	0.703
Ade	18.6	0.115	0.618

ions (Na^+) in the mobile phase to pair up with the micelles. In essence, since only a limited number of SDS molecules can be adsorbed on the stationary phase, there are a large number of non-adsorbed, free micelles in the mobile phase. As the SDS concentration increases, the positively charged ring moieties are attracted to the large number of available negatively charged micelles in the mobile phase.

When $1/k'$ values were plotted against SDS concentrations, a linear relationship was observed for the bases Cyt and Ade (Fig. 5). Although the relationship was not linear for the nucleosides with an amino group (Guo, Ado), both plots of $1/k'$ values against SDS concentrations had positive slopes. This relationship suggests that at pH 3.4 electrostatic attraction is the dominant effect in the retention of purine or pyrimidine compounds containing an amino group.

Effect of counter-ion concentration

By plotting the increase in sodium chloride concentration against k', the effect of counter-ion concentration is indicated (Fig. 6). Increased counter-ion concentration caused sharply decreased k' values of the bases Ade and Cyt and the nucleoside

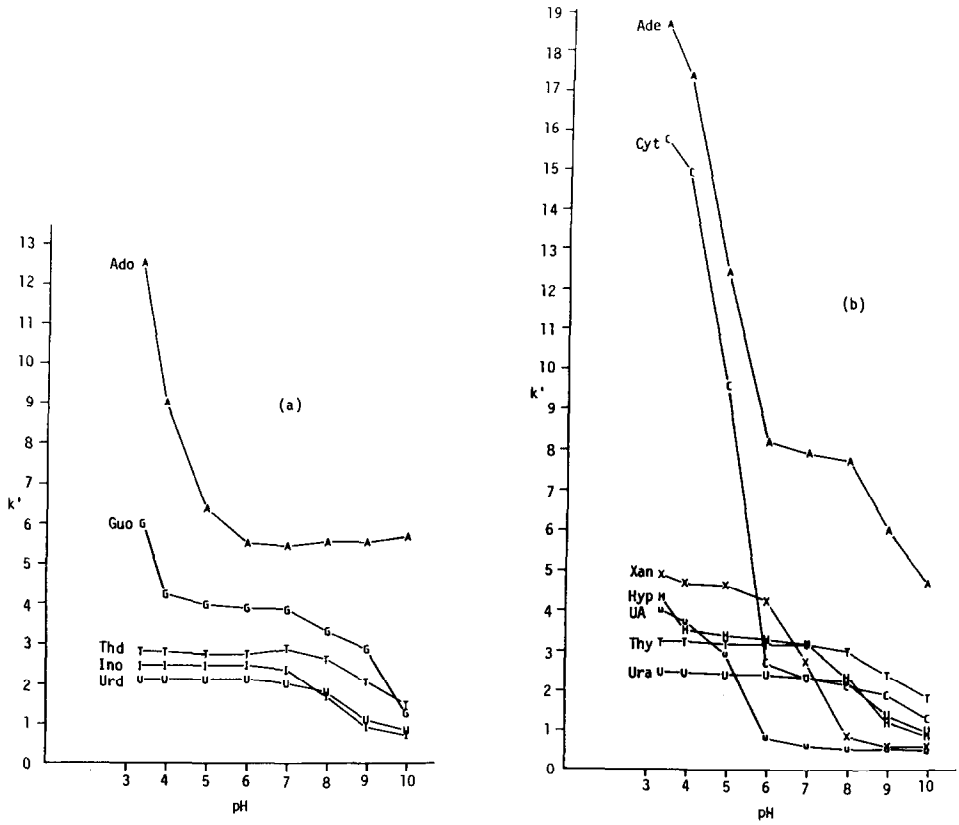

Fig. 2. Effect of pH on the capacity factors of (a) nucleosides and (b) bases. Column, 9-μm Asahipak GS 320H; mobile phase, 0.01 *M* SDS; flow-rate, 2 ml/min; temperature, ambient. Each point represents the average of three determinations.

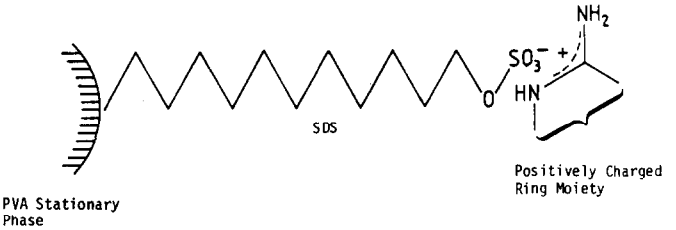

Fig. 3. Proposed electrostatic attraction of the protonated ring moiety with the negatively charged stationary phase.

Ado. At low concentrations (up to 0.01 *M*), the k' values of Hyp, Xan, UA, and Guo decreased moderately. However, the degree of reduction in retention with increasing counter-ion concentration was smaller than that with increasing SDS concentration. In addition, the elution order of Guo and Hyp changed with increasing counter-ion concentration.

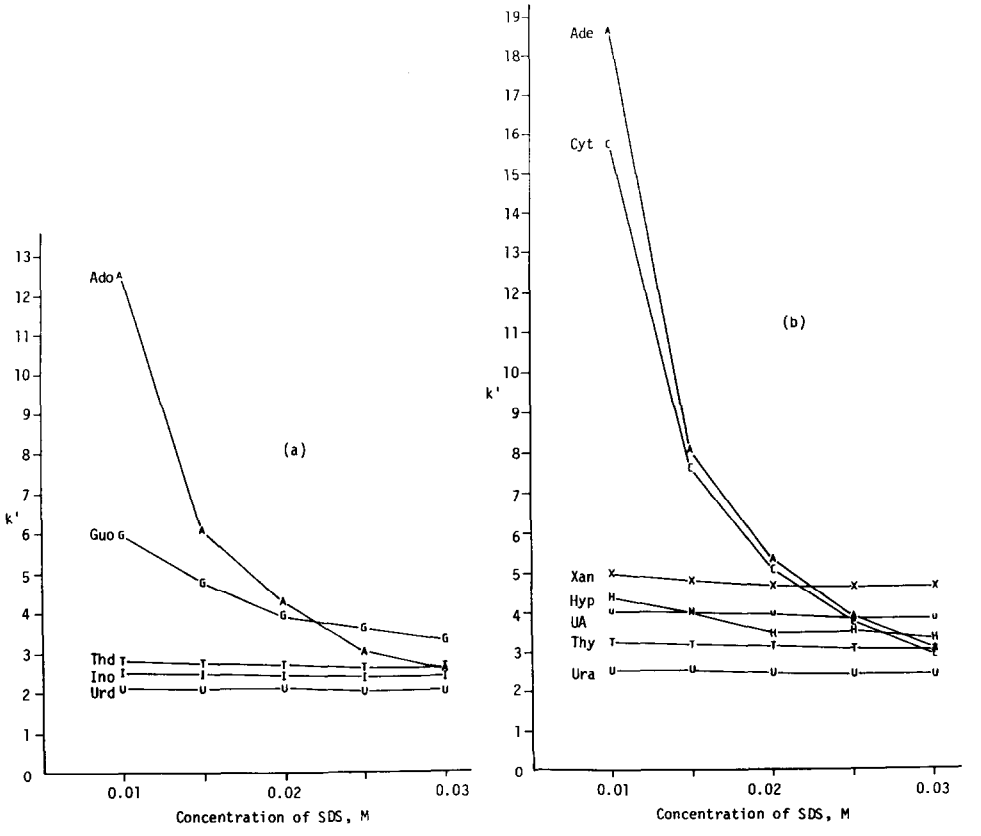

Fig. 4. Effect of SDS concentration on the capacity factors of (a) nucleosides and (b) bases. Mobile phase, aqueous SDS (pH 3.4). Other chromatographic conditions are the same as in Fig. 2. Each point represents the average of three determinations.

When capacity factors were plotted against the reciprocal of the concentration of counter ion, no linearity was observed, indicating that both electrostatic attraction and hydrophobic interaction mechanisms were operative. In addition, as the concentration of counter ion, *i.e.*, ionic strength, increases, there is a salting-out effect. The solute is then less soluble in the mobile phase, and thus the hydrophobicity of the solute is increased.

Effect of temperature

A van 't Hoff plot was constructed over a temperature range of 25–55°C with a mobile phase of 0.01 *M* SDS at pH 3.4 (Fig. 7). Linear relationships were observed for all nucleosides and bases over the full temperature range. However, the slopes of Cyt and Ade were opposite to the slopes of the bases which did not contain an amino group. In addition, the retention enthalpies, ΔH_r, for Ado and Ade from the van 't Hoff plot were -0.2 and $+0.5$ kcal/mol, respectively. These results indicate that a different retention-controlling mechanism is operative in the retention of the bases containing an amino group.

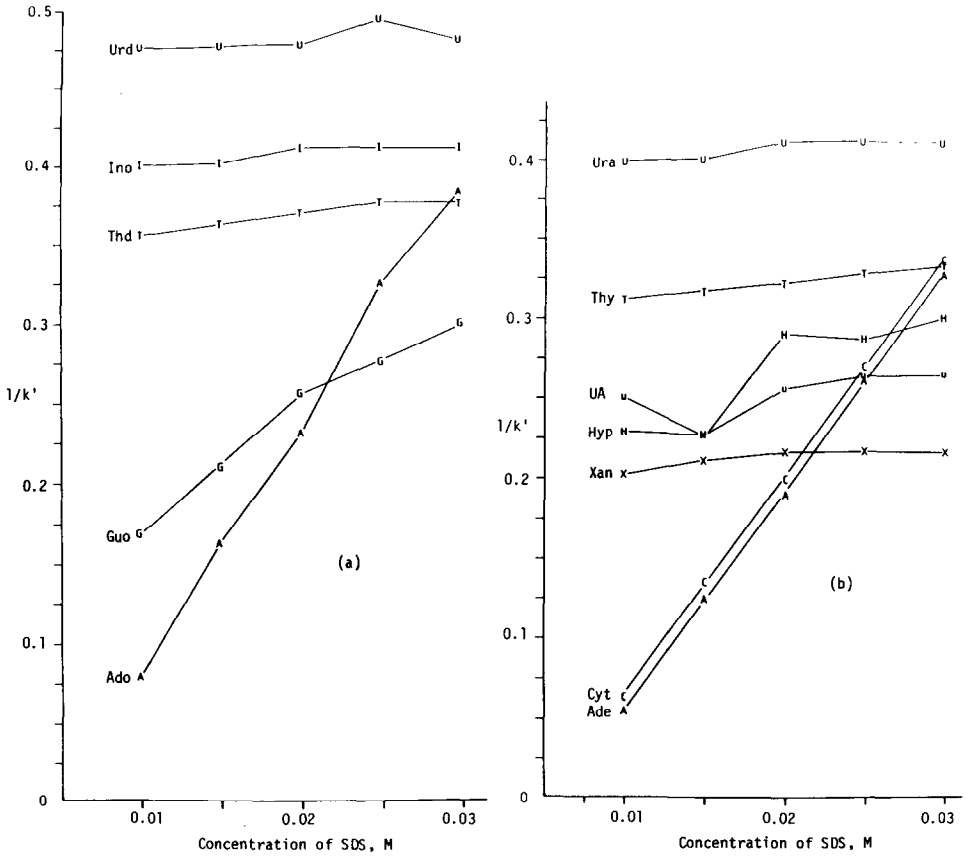

Fig. 5. A plot of SDS concentration against the reciprocal of the capacity factors of (a) nucleosides and (b) bases. All chromatographic conditions are the same as in Fig. 4. Each point represents the average of three determinations.

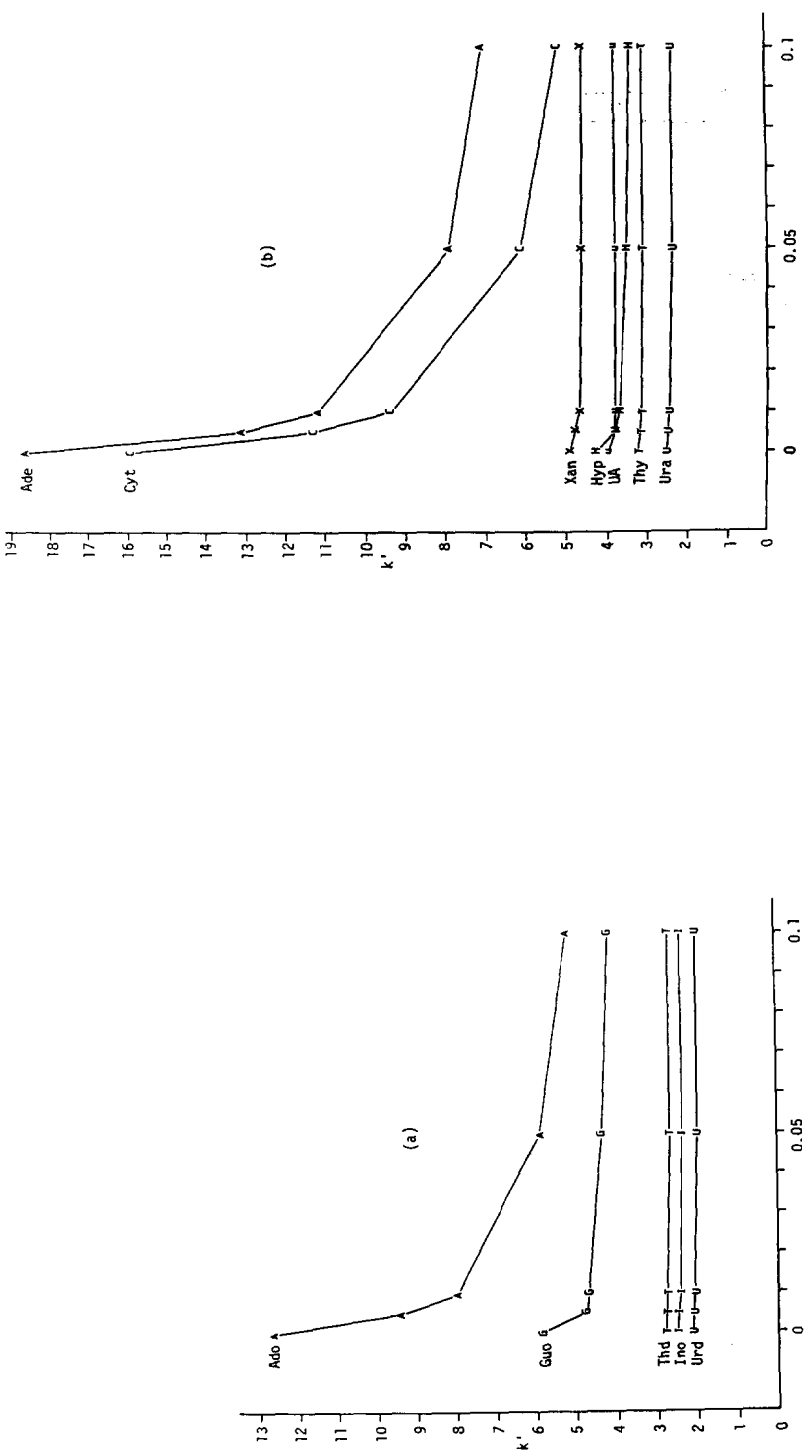

Fig. 6. Effect of counter-ion concentration on the capacity factors of (a) nucleosides and (b) bases. Concentrations of sodium chloride, 0.005, 0.01, 0.05 and 0.1 *M*; mobile phase, 0.01 *M* SDS (pH 3.4); flow-rate, 2 ml/min; temperature, ambient. Each point represents the average of three determinations.

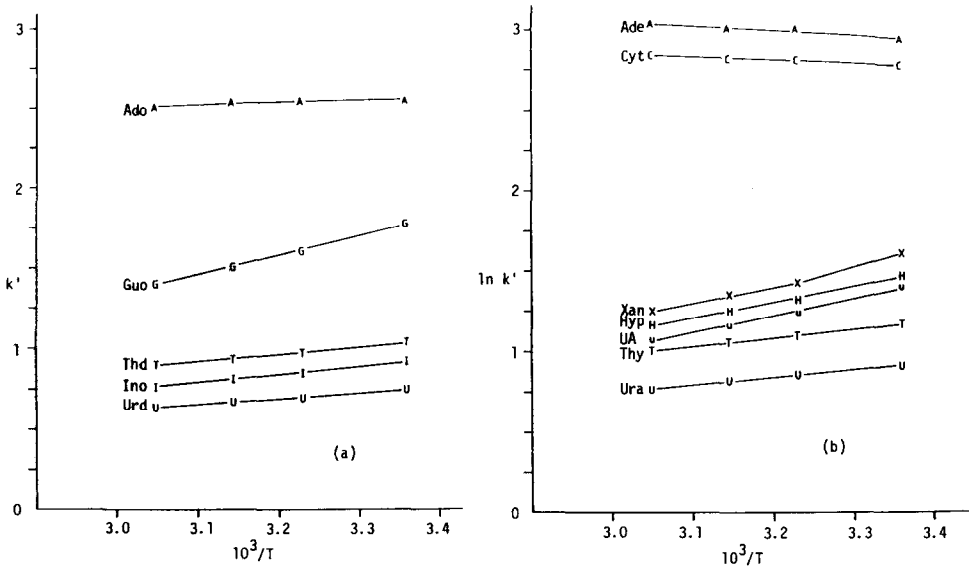

Fig. 7. Van 't Hoff plots of the capacity factors of (a) nucleosides and (b) bases. Temperature, 25, 37, 45 and 55°C; mobile phase, 0.01 *M* SDS (pH 3.4); flow-rate, 2 ml/min. Each point represents the average of three determinations.

Mechanisms of retention

Since the PVA stationary phase contains hydrophilic sites(hydroxyl groups) and hydrophobic sites (polymer matrix), it is expected that the polar head groups of SDS monomers would be adsorbed on hydrophilic sites, whereas, the hydrophobic tails of SDS monomers would be adsorbed on hydrophobic sites. As a result of our investigation of the effects of an SDS mobile phase with different types of stationary phases[36], it appears that the PVA stationary phase adsorbs the SDS monomers in two ways, *i.e.*, by hydrophilic adsorption and hydrophobic adsorption; thus, the retention behavior of nucleosides and bases on the PVA column can be controlled by hydrophobic interaction in combination with electrostatic attraction. A schematic model of hydrophobic interaction and electrostatic attraction between solutes and the modified PVA stationary phase is shown in Fig. 8.

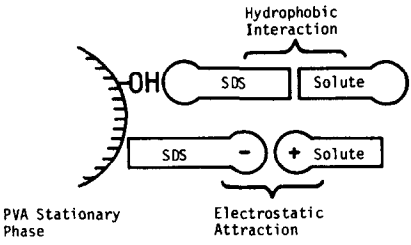

Fig. 8. A schematic model of hydrophobic interaction and electrostatic attraction between solutes and the modified PVA stationary phase.

Functional groups of purines and pyrimidines play an important role in hydrophobic interaction and electrostatic attraction. Since non-ionized bases are more hydrophobic than their respective nucleosides, which contain ribosyl or deoxyribosyl rings with several hydroxyl groups, these bases interact more with hydrophobic sites on the modified PVA stationary phase. As a result, nucleosides are eluted earlier than their respective bases (Figs. 1 and 2). Higher k' values of purine bases compared to those of the pyrimidine bases are also due to the hydrophobic interaction of the purines with the stationary phase. The additional ring in purine bases contributes to increased hydrophobicity and gives purines longer retention times than pyrimidines. The effect of hydrophobic interaction can also be observed in the elution of Ura and Thy, which have similar structures. However, Thy has a methyl group at C-5, which makes it more hydrophobic; thus, Thy is retained longer than Ura. As was discussed in the previous sections, electrostatic attraction takes place and mainly controls retention when positively charged purines and pyrimidines containing an amino group interact with the negatively charged sites on the modified PVA stationary phase.

DISCUSSION

Most of the published separations of nucleosides and bases have been achieved by RPLC on silica-based columns. In order to determine whether our micellar method was useful for the analysis of nucleosides and bases in biological matrices, the low-molecular-weight, UV-absorbing constituents of human serum were separated on the PVA column by isocratic elution with 0.01 M SDS at different pH values (Fig. 9).

Optimum separation was achieved at pH 3.4. In contrast to the RPLC gradient method, there were no interferences from tyrosine, phenylalanine, and creatinine. In addition, Urd, Hyp, and Xan, which usually overlap, and Ino and Guo, which are eluted very close together in RPLC, are well separated by the micellar method. Under the stated conditions, Ino and Ura are unresolved, and the UA peak can interfere

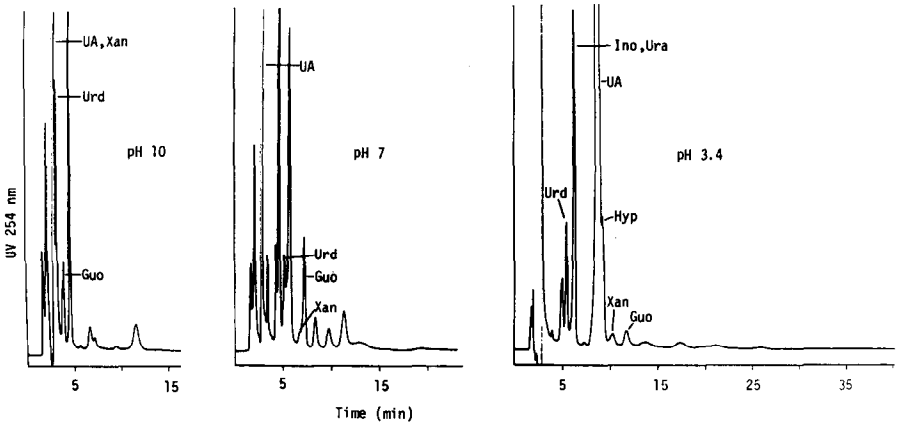

Fig. 9. Isocratic elution profiles of human serum at different pH values. Injection volume, 30 μl. Other chromatographic conditions are the same as in Fig. 1.

TABLE II

QUANTITATION AND DETECTION LIMITS OF SELECTED NUCLEOSIDES AND BASES

$n = 4$.

Compound	*Concentration (μmole/l)*	*S.D.*	*C.V. (%)*	*Literature value (μmole/l)*	*Detection limits* (μmole/l)*
Urd	8.35	0.161	1.93	0.95–8[24,40]	0.2
Guo	3.19	0.0424	1.33	0–1.98[24]	0.8
UA	202	5.10	2.52	155–429[24]	0.6
Xan	2.71	0.117	4.32	0.54–4.70[24]	0.8

* Detection sensitivity 0.005 a.u.f.s. (signal-to-noise ratio = 2).

with the Hyp peak if a large amount of UA is present. However, these peaks can be separated by adjustments in mobile phase, even though the separation was not designed for the resolution of these two compounds.

Quantitation and detection limits of Urd, Guo, UA, and Xan in human serum were investigated. The results are summarized in Table II. The values for serum constituents are in good agreement with those obtained by RPLC methods reported in the literature.

CONCLUSIONS

Micellar liquid chromatography on a PVA column was found to be quite selective and useful for the separation of some of the important nucleosides and bases found in biological matrices, especially, purines and pyrimidines which contain keto groups. The isocratic elution gave adequate separation of these compounds, compared to gradient elution with hydroorganic mobile phases. The main advantage of the method is that rapid separation of the major purines and pyrimidines containing keto groups can be obtained isocratically; thus, equilibration after each separation is unnecessary. In addition, different selectivities are found for the various nucleosides and bases; compounds that are not well separated by RPLC can be readily resolved by this method. Thus, this method can be used in conjunction with RPLC in the identification of peaks. Since it can also be optimized for any selected nuceloside or base and/or a group of these compounds, micellar liquid chromatography on a PVA column can be very useful for monitoring abnormalities in purine and pyrimidine metabolism as well as therapeutically important nucleoside and base analogues in biological fluids.

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